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Abstract: Glucans like cellulose and starch are a major source of carbon for decomposer food webs, especially during early- and intermediate-stages of decomposition. Litter quality has previously been suggested to notably influence decomposition processes as it determines the decomposability of organic material and the nutrient availability to the decomposer community. To study the impact of chemical and elemental composition of resources on glucan decomposition, a laboratory experiment was carried out using beech (*Fagus sylvatica*, L.) litter from four different locations in Austria, differing in composition (concentration of starch, cellulose and acid unhydrolyzable residue or AUR fraction) and elemental stoichiometry (C:N:P ratio). Leaf litter was incubated in mesocosms for six months in the laboratory under controlled conditions. To investigate the process of glucan decomposition and its controls, we developed an isotope pool dilution (IPD) assay using (¹³C)-glucose to label the pool of free glucose in the litter, and subsequently measured the dilution of label over time. This enabled us to calculate gross rates of glucose production through glucan depolymerization, and glucose consumption by the microbial community. In addition, potential activities of extracellular cellulases and ligninases (peroxidases and phenoloxidases) were measured to identify effects of resource chemistry and stoichiometry on microbial enzyme production. Gross rates of glucan depolymerization and glucose consumption were highly correlated, indicating that both processes are co-regulated and intrinsically linked by the microbial demand for C and energy and thereby to resource allocation to enzymes that depolymerize glucans. At early stages of decomposition, glucan depolymerization rates were correlated with starch content, indicating that starch was the primary source for glucose. With progressing litter decomposition, the correlation with starch diminished and glucan depolymerization rates were highly correlated to cellulase activities, suggesting that cellulose was the primary substrate for glucan depolymerization at this stage of decomposition. Litter stoichiometry did not affect glucan depolymerization or glucose consumption rates early in decomposition. At later stages, however, we found significant negative relationships between glucan depolymerization and litter C:N and AUR:N ratio and a positive relationship between glucan depolymerization and litter N concentration. Litter C:N and C:P ratios were negatively related to cellulase, peroxidase and phenoloxidase activities three and six months after incubation, further corroborating the importance of resource stoichiometry for glucan depolymerization after the initial pulse of starch degradation.

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Influence of litter chemistry and stoichiometry on glucan depolymerization during decomposition of beech (*Fagus sylvatica* L.) litter

Sonja Leitner^{a,b}, Wolfgang Wanek^{a,*}, Birgit Wild^a, Ieda Haemmerle^a, Lukas Kohl^a, Katharina M. Keiblinger^{b,c}, Sophie Zechmeister-Boltenstern^{b,c}, Andreas Richter^{a,d}

^a Department of Terrestrial Ecosystem Research, Faculty of Life Sciences, University of Vienna, Althanstraße 14, 1090 Vienna, Austria

^b Institute of Soil Research, Department of Forest and Soil Sciences, BOKU – University of Natural Resources and Life Sciences, Peter Jordan-Strasse 82, 1190 Vienna, Austria

^c BFW – Federal Research and Training Centre for Forests, Natural Hazards and Landscape, Seckendorff-Gudent Weg 8, 1130 Vienna, Austria

^d School of Earth and Environment, Ecosystems Research Group, The University of Western Australia, 35 Stirling Highway, Crawley, Perth, WA 6009, Australia

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ABSTRACT

Glucans like cellulose and starch are a major source of carbon for decomposer food webs, especially during early- and intermediate-stages of decomposition. Litter quality has previously been suggested to notably influence decomposition processes as it determines the decomposability of organic material and the nutrient availability to the decomposer community. To study the impact of chemical and elemental composition of resources on glucan decomposition, a laboratory experiment was carried out using beech (*Fagus sylvatica*, L.) litter from four different locations in Austria, differing in composition (concentration of starch, cellulose and acid unhydrolyzable residue or AUR fraction) and elemental stoichiometry (C:N:P ratio). Leaf litter was incubated in mesocosms for six months in the laboratory under controlled conditions. To investigate the process of glucan decomposition and its controls, we developed an isotope pool dilution (IPD) assay using ¹³C-glucose to label the pool of free glucose in the litter, and subsequently measured the dilution of label over time. This enabled us to calculate gross rates of glucose production through glucan depolymerization, and glucose consumption by the microbial community. In addition, potential activities of extracellular cellulases and ligninases (peroxidases and phenoloxidases) were measured to identify effects of resource chemistry and stoichiometry on microbial enzyme production. Gross rates of glucan depolymerization and glucose consumption were highly correlated, indicating that both processes are co-regulated and intrinsically linked by the microbial demand for C and energy and thereby to resource allocation to enzymes that depolymerize glucans. At early stages of decomposition, glucan depolymerization rates were correlated with starch content, indicating that starch was the primary source for glucose. With progressing litter decomposition, the correlation with starch diminished and glucan depolymerization rates were highly correlated to cellulase activities, suggesting that cellulose was the primary substrate for glucan depolymerization at this stage of decomposition. Litter stoichiometry did not affect glucan depolymerization or glucose consumption rates early in decomposition. At later stages, however, we found significant negative relationships between glucan depolymerization and litter C:N and AUR:N ratio and a positive relationship between glucan depolymerization and litter N concentration. Litter C:N and C:P ratios were negatively related to cellulase, peroxidase and phenoloxidase activities three and six months after incubation, further corroborating the importance of resource stoichiometry for glucan depolymerization after the initial pulse of starch degradation.

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1. Introduction

Cellulose and starch represent a major source of carbon (C) for microbial decomposer communities, constituting about one-third

* Corresponding author. Tel.: +43 (0) 1 4277 54254; fax: +43 (0) 1 4277 9542.
E-mail addresses: w_wanek@gmx.at, wolfgang.wanek@univie.ac.at (W. Wanek).

of total plant biomass (Somerville, 2006). Cellulose is the major structural component of the plant cell wall (Brett and Waldron, 1996), which can be found in nearly all plant tissues, and has therefore been denoted the most abundant biopolymer on earth (Perez et al., 2002). Starch is an osmotically inactive storage molecule, which is synthesized during photosynthesis and stored in form of starch granules in plastids (Taiz and Zeiger, 2002). Cellulose is considered more recalcitrant than starch because of its

internal composition of β -linked glucose units resulting in long fiber structures (Perez et al., 2002) and its interconnection with lignin forming the so called “lignocellulose” complex. The ratios of easily degradable to recalcitrant compounds have been reported to substantially influence the rate of litter decomposition (Aber et al., 1990; Austin and Ballare, 2010; Berg and Agren, 1984; Couteaux et al., 1995; Fioretto et al., 2005).

The enzymatic depolymerization of glucans by extracellular enzymes is considered to be the rate-limiting step in glucan decomposition (Perez et al., 2002). Extracellular enzymes are produced and excreted by microbial decomposers to depolymerize macromolecules into smaller, soluble compounds that can subsequently be taken up by microorganisms. The production of extracellular enzymes is regulated by nutrient availability and is thought to reflect the demand of the microbial community for nutrients and energy (Sinsabaugh et al., 2009). As enzyme production requires nutrients, especially nitrogen (N) (Schimel and Weintraub, 2003), the elemental composition of plant litter (i.e., its elemental stoichiometry) may have a major impact on decomposition rates. Activities of extracellular C-acquiring enzymes like cellulases and amylases have previously been suggested to be N-limited (Berg, 2000; Berg and Matzner, 1997; Fog, 1988) and should therefore be enhanced if N is readily available, resulting in higher glucan depolymerization rates in litter with low C:N ratios.

Up to now, decomposition of cellulose and starch in plant litter could only be examined by observations of the decrease in the respective pool size compared to absolute litter mass loss (e.g., Fioretto et al., 2005; Papa et al., 2008; Sariyildiz and Anderson, 2003; Tagliavini et al., 2007), or by measuring potential activities of glucan-depolymerizing enzymes i.e. cellulases and amylases (e.g., Fioretto et al., 2005; Papa et al., 2008) via photometric and fluorimetric assays (Koenig et al., 2002; Marx et al., 2001; Sinsabaugh et al., 1999). However, changes in pool sizes only provide insight into long-term depolymerization rates, and the determination of potential enzyme activities has previously been criticized as they do not represent the *actual rates* of decomposition, which are dependent on concentration and accessibility of substrates as well as on enzyme activity and environmental conditions (Wallenstein and Weintraub, 2008). To overcome these challenges, we have established a new method to estimate actual glucan depolymerization rates based on the isotope pool dilution (IPD) technique (Di et al., 2000; Kirkham and Bartholomew, 1954; Wanek et al., 2010). We added ^{13}C -glucose to label the pool of free glucose (i.e., glucose that is not bound in starch, cellulose or other compounds) in decomposing litter and subsequently determined the rate at which the label was diluted and glucose concentration changed. This allowed us to estimate gross rates of glucose production, which we consider to be derived mainly from enzymatic glucan depolymerization.

We used this new approach to investigate how litter chemistry and elemental stoichiometry control glucose production by glucan depolymerization in decomposing litter, and how glucan depolymerization is affected by the activity of extracellular enzymes. We hypothesized that (i) the rates of glucose production are positively correlated with litter N concentration (and negatively with litter C:N ratios), due to an increased resource allocation to C-acquiring enzymes with increasing litter N availability, and (ii) that glucan depolymerization is positively correlated with substrate concentration (starch, cellulose) and activities of the respective extracellular enzymes involved in glucan decomposition.

To examine the controls of litter chemistry, including the concentrations of starch, cellulose and lignin as well as the stoichiometry of C and N availability, on glucan depolymerization, we conducted a laboratory incubation experiment under controlled conditions using beech (*Fagus sylvatica* L.) litter of varying

elemental stoichiometry (C:N and C:P ratios) and chemical composition. The litter had been sterilized by γ -irradiation and re-inoculated with a beech forest soil inoculum (organic horizon) to establish a comparable initial microbial community for each litter type, and was then incubated for six months in mesocosms at constant temperature and humidity (Wanek et al., 2010).

2. Material and methods

To clarify the use of terminology we define carbohydrates, being synonymous with saccharides, as organic compounds with the general formula $\text{C}_m(\text{H}_2\text{O})_n$, i.e. polyhydroxy aldehydes or polyhydroxy ketones. Saccharides are divided into monosaccharides ($(\text{C}_6\text{H}_{10}\text{O}_5)_n$ with $n > 3$, e.g. glucose, fructose, xylose, arabinose), disaccharides (e.g. sucrose), oligosaccharides and polysaccharides. The term sugars is commonly used for mono- and disaccharides. Oligosaccharides typically contain between three and ten monosaccharide units, and polysaccharides more than ten units. The latter are an important class of biopolymers, including the glucose homo-polymers starch and cellulose (glucans, i.e. polysaccharides of α -glucose monomers linked by α - or β -glycosidic bonds) and the hetero-polymers hemicellulose (e.g. xylans, arabinoxylans, galactomannans). Hemicelluloses in beech contain only traces of glucose (<1%) and are mainly composed of xylose (65%), arabinose (20%) and galactose (15%), and therefore are not subsumed under glucans (Schadel et al., 2010). Free glucose comprises the free, mostly cytosolic and vacuolar pool of unbound dissolved glucose, as contrasted by bound glucose such as that contained in disaccharides (e.g. sucrose, maltose, cellobiose), oligosaccharides (e.g. raffinose) or polymeric glucose (starch, cellulose). Decomposition of homo-polymeric glucans therefore releases a range of glucose-based oligosaccharides, disaccharides of glucose (maltose, cellobiose) and ultimately free glucose.

2.1. Litter decomposition experiment

A litter decomposition experiment was carried out over six months to explore the impact of litter quality on glucan decomposition. Freshly fallen beech (*F. sylvatica* L.) leaf litter with different litter chemistry and elemental stoichiometry (referred to as ‘litter type’) was collected in autumn 2008 from four sampling sites in Austria: Achenkirch (A), Klausenleopoldsdorf (K), Ossiach (O) and Schottenwald (S). Site characteristics and preparation of the litter were described by Wanek et al. (2010). In short, leaves were dried at 40 °C for 48 h, shred to pieces between 1 and 20 mm and sterilized twice with gamma rays of 35 kGy with one week between irradiation events. Then all litter types were inoculated with a suspension of an O-horizon:litter mixture (1:1 (w:w)) collected from Klausenleopoldsdorf in December 2008 to obtain an identical initial microbial community structure on all four litter types. For the inoculation, each litter type and the inoculum suspension amounting to 1.5% dry weight of litter were thoroughly mixed. After homogeneous distribution of the inoculum, mesocosms ($n = 5$), which had been prepared from PVC plastic tubing (10 cm length, 12.5 cm diameter), were filled with 60 g of litter fresh weight for all four litter types (A, K, O and S) and three harvest time points (two weeks, three and six months after inoculation), giving 60 mesocosms in total. Mesocosms were placed on humid sponge cloth in a climate chamber at 15 °C and watered weekly with autoclaved tap water to keep the water content stable at 60% fresh weight. Additional mesocosms were prepared for each harvest and litter type to determine the size of the free glucose pool one week before the actual harvests. The litter from each mesocosm was homogenized before analysis by putting it into a plastic bag and thoroughly mixing it.

2.2. Isotope pool dilution assay

To determine rates of gross glucan depolymerization and gross glucose immobilization, a new isotope pool dilution (IPD) assay was developed. Isotope pool dilution is a common method to determine gross transformation rates of N, P and S in soil (Di et al., 2000) by labeling a target pool and subsequently measuring the dilution of the label and the change in pool size. We developed an IPD method using ^{13}C -labeled glucose (99 atom % $^{13}\text{C}_6$ D-glucose, Isotec Inc.) as a tracer. What this IPD assay therefore measures are gross influx rates into the labeled free glucose pool (which is by glucose production from glucan decomposition) and gross efflux rates (which in this experiment is mainly due to microbial uptake/immobilization). Glucose production from glucan decomposition leads to input of unlabeled glucose and therefore to isotope pool dilution of the free glucose pool, while microbial uptake/immobilization consumes glucose with the ^{13}C : ^{12}C ratio as present at any time point in the free glucose pool. For the assay, 1.5 g of litter fresh weight were filled into 50 ml HDPE centrifuge tubes in triplicates. To start the assay, a maximum of 50% of the free glucose pool in the sample was added in form of ^{13}C -labeled glucose (30 atom% ^{13}C) dissolved in high purity water (MilliQ, >18.2 MOhm, Millipore), ranging from 0.01 to 5 mg ^{13}C -Glc l^{-1} in 5 ml MilliQ water. The size of the free glucose pool was determined for each litter type prior to the start of the IPD assay. The vials were shaken vigorously to distribute the label homogeneously. The amount of liquid added via the tracer solution was selected to form a thin water film on the leaf particles that assured a homogeneous tracer distribution without causing anoxic conditions. After shaking the vials, they were re-opened, sealed loosely with cotton wool to enable gas exchange and then incubated at 15 °C for 30, 60 and 120 min, respectively. Following the incubation, samples were extracted with 30 ml of MilliQ water at room temperature on a laboratory shaker for 15 min and centrifuged for 5 min at 10,845 g. The supernatant was then decanted into 30 ml syringes that contained a plug of cotton wool on the bottom to prevent blockage of the luer taper and subsequently filtered over a carbohydrate-free glass microfiber filter (GF/C, Whatman, 1.2 μm) inside a filter device (Swinnex, Millipore). The procedure of filtration was considered to be sufficient for stopping the assay because exo-cellulases bind firmly onto cellulose fibers and are removed by filtration, terminating ongoing glucan degradation. A test of cellulase (β -1,4-cellobiosidase) activity showed no residual enzyme activity in the filtrates. Furthermore, glucose concentration and $\delta^{13}\text{C}$ did not decrease after filtration, demonstrating that no microbial consumption and glucan depolymerization took place in the filtrates (data not shown).

2.3. Isolation of glucose from litter

Immediately after filtration, the solution was applied to coupled cation and anion exchange cartridges (OnGuard II H, volume 1 cc, H^+ form, on top of OnGuard II A, volume 1 cc, bicarbonate form; both from Dionex) which had been soaked by flushing with 10 ml of MilliQ water for two hours prior to sample application. After the sample solution was passed slowly through the ion exchange cartridges, they were eluted with 5 ml of MilliQ water to collect non-adsorbed neutral compounds (including glucose). The flow-through was collected and transferred into 250 ml vacuum proof round bottom flasks, frozen at -20°C and freeze-dried for 24 h. The residue was dissolved in 3 ml of MilliQ water, transferred into 20 ml HDPE vials, frozen again and freeze-dried over night. The dried extract was dissolved in 0.5 ml MilliQ water and stored frozen until analysis.

2.4. Isotopic analysis

The amount and $\delta^{13}\text{C}$ value of glucose in the samples was measured via compound-specific isotope analysis on a high performance liquid chromatography-isotope ratio mass spectrometer (HPLC-IRMS) system as described by Wild et al. (2010). The HPLC system consisted of an ICS3000 pump, an AS50 autosampler with a 25 μl injection loop and an Ultimate 3000 column compartment (all provided by Dionex). The separation column was a HyperREZ XP Carbohydrate Ca^{2+} 8 μm column (Thermo Fisher Scientific, USA), run at 85 °C with 0.5 ml min^{-1} MilliQ water as eluent. The HPLC was connected to the IRMS (Finnigan Delta V Advantage Mass Spectrometer, Thermo Fisher Scientific, USA) via a Finnigan LC IsoLink Interface (Thermo Fisher Scientific, USA), where the glucose was oxidized to CO_2 via acid persulfate digestion inside an oxidation reactor at 99.9 °C. As oxidant, a 0.5 M solution of sodium persulfate (sodium peroxodisulfate purum p.a., $\geq 99\%$, Fluka, Sigma–Aldrich) and 1.7 M phosphoric acid (orthophosphoric acid puriss. p.a., crystallized, $\geq 99\%$, Fluka, Sigma–Aldrich) were added to the column effluent at a flow rate of 50 $\mu\text{l min}^{-1}$ each. In a gas separation unit, the CO_2 was transferred over gas-permeable membranes to a counter flow of helium as carrier gas. This gas stream was dried over Nafion tubes and before entering the IRMS via an open split, excess oxygen was removed as described by Hettmann et al. (2007) inside a reduction reactor to improve both filament lifetime and reproducibility of the analysis.

2.5. Spiking of low concentration samples

The limit of isotope determination of glucose at precision better than 0.25‰ (SD) with the HPLC-IsoLink-IRMS system was approximately 20 mg Glc l^{-1} . Samples with glucose concentrations below this limit had to be measured through spiking of the samples. A standard stock solution with a concentration of 10 g l^{-1} D-glucose (Merck, Vienna, Austria) in MilliQ water was prepared and a working solution was prepared freshly every day by diluting the stock solution 1:10. Then, 10 μl of the working solution were pipetted into 250 μl glass inserts for GC vials and 90 μl sample were added. The concentration of the standard in the spiked sample therefore constituted 100 mg Glc l^{-1} . The $\delta^{13}\text{C}$ value of the spiked sample was then measured on the HPLC-IRMS system. Additionally, the glucose concentration of unspiked samples was determined on a high performance anion exchange chromatography-pulsed amperometric detection system (HPAEC-PAD), which has a much lower detection limit than the HPLC-IRMS system (0.024 mg l^{-1}). The HPAEC-PAD system consisted of an ICS3000 SP-1 Pump, an AS50 Autosampler with a 10 μl injection loop and an ICS3000 DC-2 Detector/Chromatography Module (all provided by Dionex, Vienna, Austria). As separation column a CarboPac PA20 (3 \times 150 mm Analytical Column with a CarboPac PA20, 3 \times 30 mm Guard Column, Dionex) was run with 0.5 ml min^{-1} 20 mM NaOH as eluent. For calibration, the glucose stock solution was used in concentrations between 0.1 and 50 mg l^{-1} . The $\delta^{13}\text{C}$ value of the glucose in the sample could then be determined using an isotopic mixing model (1):

$$\delta_{\text{sample}} = \frac{c_{\text{all}} \cdot \delta_{\text{all}} - c_{\text{spike}} \cdot \delta_{\text{spike}}}{c_{\text{sample}}} \quad (1)$$

$$c_{\text{all}} = c_{\text{sample}} + c_{\text{spike}} \quad (2)$$

where c_{sample} is the concentration of glucose in the sample as measured by the HPAEC-PAD system. c_{spike} is the concentration of the glucose standard, which was 100 mg l^{-1} , and c_{all} is the

calculated concentration of the spiked sample as shown in Eq. (2). δ_{spike} and δ_{all} were measured with the HPLC-IRMS system. As standards for calibration on the HPLC-IRMS, the glucose stock solution was used in concentration of 100 and 150 mg l⁻¹ and injected at least eight times each, four times at the beginning and four times at the end of a measurement session, with additional injections of the 100 mg l⁻¹ standard every 15 samples. For δ_{spike} , the mean value of the 100 mg l⁻¹ standard was taken. Eq. (1) was then used to calculate the δ_{sample} .

2.6. Calculations

To calculate gross rates of glucan depolymerization (GD, Eq. (3)) as influx into the soluble glucose pool, and glucose consumption (GC, Eq. (4)) as efflux from the soluble glucose pool (both given in $\mu\text{g Glc-C g}^{-1} \text{ d.w. d}^{-1}$), we adopted the equations of pool dilution theory by Kirkham and Bartholomew (Di et al., 2000; Kirkham and Bartholomew, 1954) as follows:

$$\text{GD} = \frac{C_{t2} - C_{t1} \cdot 60 \cdot 24 \cdot \frac{\ln(\text{APE}_{t1}/\text{APE}_{t2})}{\ln(C_{t2}/C_{t1})}}{t2 - t1} \quad (3)$$

$$\text{GC} = \frac{C_{t1} - C_{t2} \cdot 60 \cdot 24 \cdot \left(1 + \frac{\ln(\text{APE}_{t2}/\text{APE}_{t1})}{\ln(C_{t2}/C_{t1})}\right)}{t2 - t1} \quad (4)$$

$$\text{APE} = \text{atom}\%^{13}\text{C}_{\text{sample}} - \text{atom}\%^{13}\text{C}_{\text{background}} \quad (5)$$

$$\text{atom}\% = \frac{R_{V\text{-PDB}} \cdot \left(\delta^{13}\text{C}_{\text{sample}}/1000 + 1\right)}{1 + R_{V\text{-PDB}} \cdot \left(\delta^{13}\text{C}_{\text{sample}}/1000 + 1\right)} \cdot 100 \quad (6)$$

where $t1$ and $t2$ are the times of stopping the pool dilution assay (30 and 120 min), C_{t1} and C_{t2} are the amounts of glucose-C at time $t1$ and $t2$ ($\mu\text{g Glc-C g}^{-1} \text{ d.w.}$), and APE_{t1} and APE_{t2} are the values of glucose-¹³C atom percent excess (%) at time $t1$ and $t2$, calculated as shown in Eq. (5). Atom percent excess was calculated from the amount of ¹³C atom% in the labeled sample corrected for the background natural ¹³C abundance (about 1.08 atom%). Eq. (6) was used to calculate atom% from the measured $\delta^{13}\text{C}$ values of the sample, where R_{sample} and $R_{V\text{-PDB}}$ are the ratios of ¹³C:¹²C of the sample and the international standard V-PDB (Vienna Pee Dee Belemnite), respectively, with $R_{V\text{-PDB}}$ being 0.0112372.

2.7. Considerations on the isotope pool dilution technique

While labeling the small litter glucose pool, one faces two divergent problems: to add only as much labeled glucose as necessary to obtain an isotope signal without disturbing the natural processes too much, but to adjust the amount of added label to get measurable isotope signals for the HPLC-IRMS system within the linear measurement range. To address the first point, we determined the glucose pool size prior to the IPD assay and added 50% of the glucose pool as labeled substrate. Addressing the second point was more complicated due to strong decreases in glucose concentrations in litter over time. We had to dilute the ¹³C-label by adding 30% ¹³C-glucose and 70% ¹²C-glucose to give an in situ enrichment of glucose after label addition of approximately 10 atom% ¹³C, otherwise the ¹³C-signal would be above the linear range of the HPLC-IRMS system. At the harvests after 3 and 6 months, however, glucose concentrations of the litter were low, the added glucose label was consumed faster and we had to add a spike of unlabeled glucose before accurately measuring the ¹³C:¹²C ratio of free glucose by IRMS. When performing the IPD, three stopping times were used, after 30', 60' and 120'. Between 30' and 60', mean

label consumption ranged between 10% and 60% at harvest one and between 10% and 75% at harvests two and three. Between 30' and 120', label consumption ranged between 20% and 80% at harvest one and between 60% and 90% at harvests two and three. It can be a problem for precise IPD measurements if more than 80 or 90% of the label is consumed because then influx and efflux rates are likely to be underestimated or the isotope ratio methods are not sensitive enough to see any isotope pool dilution. We therefore calculated rates between all time points (30–60', 60–120' and 30–120') and then decided to use the longest interval (30–120') to calculate the rates because (a) the equations for IPD are problematic if differences between the concentrations and isotopic signatures are small and we therefore got a lot of negative rates or equation failures if the short intervals (30–60' and 60–120') were used, and (b) because rates calculated based on the long interval (30–120') averaged across those measured for the two short intervals. On average rates calculated for 30–120' were 39% lower than those derived from 30 to 60'. The HPLC-IRMS system used has a very low detection limit and determines carbon isotope ratios close to natural abundance very precisely ($<1 \mu\text{g glucose-C sample}^{-1}$) without hampering the calculation of isotope fluxes and dilution; we therefore decided to use the longer interval although a large part of the label was consumed.

2.8. Potential enzyme activities

The potential activity of β -1,4-cellobiosidase as a representative of the large class of cellulases ('cellulase') was determined using a microplate fluorimetric assay based on MUF- β -D-cellobioside (4-methylumbelliferyl- β -D-cellobioside, 0.5 mM in 100 mM sodium acetate buffer) (Kaiser et al., 2010; Marx et al., 2001). The activities of β -1,4-cellobiosidase and β -glucosidase have previously been used as a proxy for total cellulase activity in soils (e.g., Geisseler and Horwath, 2009; Kaiser et al., 2010; Sinsabaugh et al., 2009). β -1,4-cellobiosidase is an exo-cellulase and releases the disaccharide cellobiose from cellulose and cellotetraose, which then is cleaved by β -glucosidase into two glucose monomers to be taken up by microbes (Perez et al., 2002). Activities of oxidative enzymes (peroxidase and phenoloxidase) were measured photometrically according to standard assays (Sinsabaugh et al., 1999) with small modifications as described by Kaiser et al. (2010). As substrate, L-3,4-dihydroxyphenylalanine (L-DOPA, 20 mM) was used for both assays of oxidative enzymes.

2.9. Litter and microbial stoichiometry, respiration and litter chemistry

Litter C (C_{lit}) and N (N_{lit}) content were measured after drying at 80 °C and grinding in a ball mill with an elemental analyzer (EA 1110, CE Instruments). Phosphorus content of the ground litter (P_{lit}) was determined after acid digestion (Henschler, 1988) by inductively coupled plasma atomic emission spectrometry (ICP-AES). Microbial biomass C (C_{mic}) and N (N_{mic}) content were estimated in fresh litter by the chloroform fumigation-extraction technique (Schinner et al., 1996) using a TOC/TN analyzer (TOC-VCPh and TNM-1, Shimadzu). Ratios of litter and microbial C:N, C:P and N:P as well as litter cellulose:N and AUR:N were calculated on a mass basis. C:N_{imbalance} as a measure of the imbalance between microbial and litter stoichiometry was calculated as follows:

$$\text{C:N}_{\text{imbalance}} = \frac{\text{C:N}_{\text{litter}}}{\text{C:N}_{\text{microbe}}} \quad (7)$$

Litter respiration was determined using an infrared CO₂ gas analyzer (EGM-4, PP systems) connected to a soil respiration

chamber (SRC-1 Soil Respiration Chamber, PP systems) which was placed on top of the mesocosm and the bottom was closed with a gas tight lid. Carbon dioxide concentration in the headspace was continuously recorded at 5-s intervals for 70 s, and the averaged CO₂ increase per second was used to calculate respiration rate. Respiration was measured one day prior to the harvesting of the mesocosms. Additionally, respiration was monitored throughout the entire 6 months incubation period in weekly respiration measurements using the same system (Keiblinger KM, unpublished data).

Starch content was determined as described by Goettlicher et al. (2006) by determination of glucose released after addition and incubation with heat-stable α -amylase (Sigma–Aldrich, Vienna, Austria) from *Bacillus licheniformis* (500 U ml⁻¹ MilliQ water) and amyloglucosidase (Roche Diagnostics, Vienna, Austria) from *Aspergillus niger* (10 U in 0.5 ml 20 mM sodium acetate buffer, pH 4.6) by HPAEC-PAD as described above. Cellulose and acid unhydrolyzable residue (AUR) content were determined following a modification of the acid detergent method (Rowland and Roberts, 1994) as published in the manual “Use of isotope and radiation methods in soil and water management and crop nutrition” by the International Atomic Energy Agency (<http://www-pub.iaea.org/MTCD/publications/PDF/TCS-14.pdf>). Aliquots of finely ground plant litter (0.5 g, dried at 60 °C) were weighed into F57 ANKOM filter bags for fiber analysis (ANKOM technology, Macedon, NY, USA) of known dry weight and heat sealed. In the first step hemi-celluloses, protein and starch were removed by extracting the plant material for 1.5 h in a boiling sulfuric acid solution (0.5 M) containing cetyltrimethylammonium bromide (CTAB, 20 g l⁻¹), with 100 ml CTAB/sulfuric acid per bag. The bags were washed under running demineralized water until no more foam developed, then with boiling MilliQ water until the pH was neutral, and then five times with acetone. The bags were then dried at 60 °C over night and re-weighed. The residue in the bags now consisted of acid detergent fiber (ADF) containing cellulose, AUR and ash. In a second step following the acid detergent extraction, cellulose was removed by an acid hydrolysis with 72% sulfuric acid (20 ml per sample) for 3 h at room temperature. The bags were washed again as described after the CTAB/sulfuric acid treatment, dried at 60 °C over night, re-weighed and the cellulose content was determined by difference and calculated as % initial sample dry weight. Finally, the filter bags containing the remaining fraction (AUR and ash) were ashed at 515 °C for 3 h in porcelain cups, re-weighed and the amount of AUR (% dry weight) was calculated by subtraction of the ash content from the remaining mass after cellulose removal. Given critiques on the unspecificity of the acid detergent method for lignin determination, i.e. the AUR fraction contains lignin, cutin, fatty acids and others, we tested the macromolecular composition of decomposing litter also using pyrolysis GCMS using a GSG-Pyromat (GSG Mess- und Analysegeräte) coupled to a Thermo Trace GC and a DSQ MS detector (Thermo Scientific). Organic material was pyrolyzed at 590 °C and pyrolysates separated on a Carbowax column (Supelcowax 10, Sigma–Aldrich) with a linear temperature gradient from 50 to 260 °C in 30 min. Peaks were assigned based on NiSt 05 MS library and comparison with reference materials measured. Pyrolysis products were then assigned to their substances of origin in accordance with literature and summed up for glucan and lignin fractions (Schellekens et al., 2009).

2.10. Statistical analyses

Significant differences ($p < 0.05$) between litter types and harvests were analyzed by two-way ANOVA followed by Fisher's LSD post-hoc test or, if sample sizes were unequal, Scheffé post-hoc test. Kolmogorov-Smirnov-test was performed to test for normal

distribution and Levene's test to test for homogeneity of variance. Data points with a distance from the arithmetic mean of more than twice the standard deviation were defined as outliers and excluded, and if necessary, the data was $\log(x + 1)$ -transformed to obtain normal distribution and homogeneity of variances. Relations between gross rates and litter chemistry and elemental stoichiometry as well as enzymatic activities were examined through simple Pearson correlation analysis on untransformed data. Statistical analyses were performed with Statgraphics 5.0 (Statistical Graphics Inc.) and Statistica 7.1 (StatSoft Inc.).

3. Results

3.1. Litter chemistry and elemental stoichiometry

Litter chemistry was significantly affected by harvest and litter type (Table 1). As expected, starch content (Fig. 1) decreased rapidly in the first three months from initial concentrations ranging between 0.11 and 0.29% d.w. to between 0.06 and 0.18% d.w. at harvest two, but did not further decrease between harvests two and three. Cellulose concentration (Fig. 1) ranged from 18.0 to 20.7% d.w. in the initial litter, then increased in the first three months of decomposition to values between 19.7 and 25.8% d.w., and then slightly decreased until finally reaching values between 18.1 and 24.4% d.w. after six months. Acid unhydrolyzable residue (AUR) concentrations (Fig. 1) were lowest in litter collected from Ossiach and Schottenwald, which also had the lowest AUR:N ratios (11.3–19.6). The Klausenleopoldsdorf and Achenkirch litter had the highest AUR:N ratios, ranging from 16.0 to 37.7. Cellulose and AUR content showed a strong positive relationship ($r = 0.79$, $p < 0.001$, Supplementary Table 2). In litter from Klausenleopoldsdorf and Achenkirch, AUR concentrations increased during the first three months of the experiment, and then only slightly decreased reaching final values around 26% d.w. after six months of litter decomposition. The large increase in the proportion of cellulose and AUR during the incubation may be due to the loss of mass

Table 1

Significance of effects of litter type and harvest and their interactions on C:N, C:P and N:P ratios of the litter, concentrations of free glucose, starch, cellulose and AUR (acid unhydrolyzable residue), mean residence time (MRT) of free glucose, gross rates of glucan depolymerization and glucose consumption, and cellulase, peroxidase and phenoloxidase activities assessed by two-way analysis of variance (ANOVA). Data given are F -ratios and p -values (95% confidence level) and include values from three harvests (2 weeks, 3 and 6 months) and four litter types (Achenkirch, Klausenleopoldsdorf, Ossiach and Schottenwald). Units for rates are $\mu\text{g C g}^{-1} \text{ d.w. d}^{-1}$; units for glucose concentrations are $\mu\text{g Glc-C g}^{-1} \text{ d.w.}$, units for MRT glucose are d; units for starch, cellulose and lignin concentrations are % d.w. and for enzyme activities $\text{nmol g}^{-1} \text{ d.w. min}^{-1}$.

	Harvest		Litter type		Harvest \times litter type	
	F	p	F	p	F	p
C:N _{lit}	8.45	0.0007	369.99	<0.0001	1.58	0.1749
C:P _{lit}	1.25	0.2958	251.45	<0.0001	3.10	0.0120
N:P _{lit}	3.37	0.0425	184.66	<0.0001	1.82	0.1152
Glucose concentration	112.70	<0.0001	26.90	<0.0001	16.43	<0.0001
MRT Glucose	230.31	<0.0001	48.01	<0.0001	28.01	<0.0001
Starch concentration	51.08	<0.0001	35.94	<0.0001	7.61	<0.0001
Cellulose concentration	29.20	<0.0001	24.84	<0.0001	3.41	0.0079
AUR concentration	20.18	<0.0001	37.11	<0.0001	4.56	0.0012
Gross glucan depolymerization	4.21	0.0216	10.35	<0.0001	6.47	0.0001
Gross glucose consumption	22.22	<0.0001	22.86	<0.0001	10.01	<0.0001
Cellulase activity	108.53	<0.0001	36.88	<0.0001	10.19	<0.0001
Peroxidase activity	8.44	0.0007	32.08	<0.0001	1.51	0.1962
Phenoloxidase activity	31.71	<0.0001	54.31	<0.0001	2.55	0.0317

Bold values indicate significant effects of litter type or harvest or of their interactions ($p < 0.05$).

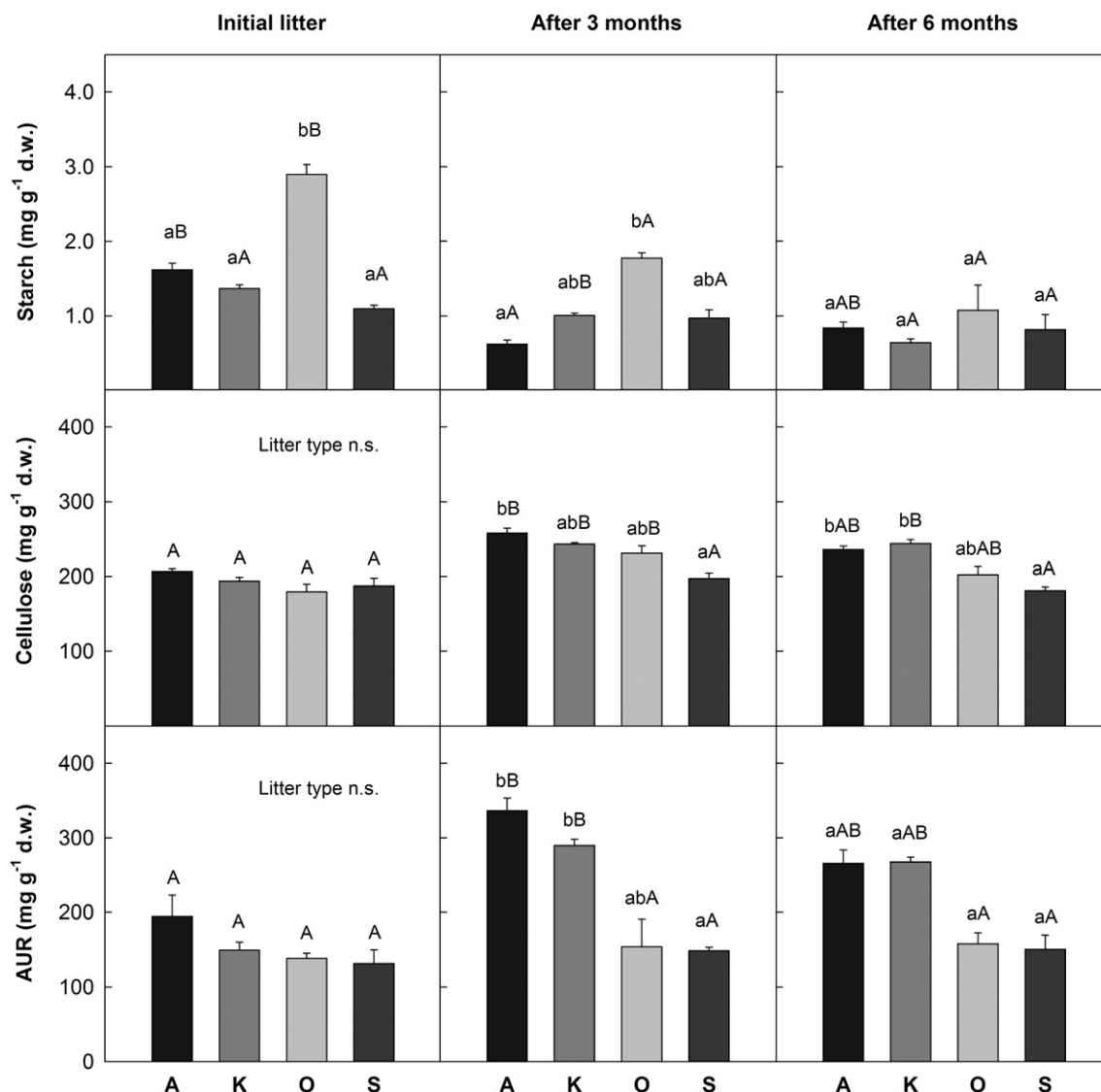


Fig. 1. Litter concentrations of starch, cellulose and acid unhydrolyzable residue (AUR) in the initial litter and after 3 and 6 months of decomposition, respectively (A, Achenkirch; K, Klausenleopoldsdorf; O, Ossiach; S, Schottenwald). Given are means of 5 mesocosms \pm SE with exception for the initial litter ($n = 4$). Lower case letters indicate significant differences between litter types, upper case letters indicate significant differences between harvests (two-way ANOVA followed by Scheffé test, $p = 0.05$).

during decomposition of the litter, due to accumulation of microbial products or be related to inaccuracies of the acid detergent methodology used to measure cellulose and AUR. Mass loss after 3 and 6 months was small, ranging between 1.2% and 2.2% of initial mass after 3 months and between 2.8% and 4.6% after 6 months. Mass loss therefore cannot account for the large increase in AUR and cellulose concentration. The technique to quantify AUR and cellulose using solvent/acid extractions has been criticized for its unspecificity (Hatfield and Fukushima, 2005). PyGCMS is much more amenable to analyze the macromolecular composition of environmental samples such as soils and litter, where pyrolysates peaks are quantified and assigned by mass spectrometry to major C fractions (glucan, lignin, protein, fatty acid etc). Lignin contents by pyGCMS initially ranged between 28.9% (A), 29.9% (K), 30.5% (S) and 31.2% (O) while carbohydrates (glucan) comprised 25.9% (A), 26.1% (K), 26.9% (S) and 29.2% (O) of dry matter. PyGCMS did not show a consistent change in lignin over the first three or six months or if an increase was found it was marginal (A -0.2% , K $+0.7\%$, O $+1.7\%$, S $+2.9\%$) compared to the increases found with solvent/

acid extractions. Cellulose decreased slightly (by 1.5–2.4%) in two litter types (Ossiach, Klausenleopoldsdorf) but not in the others. Moreover, pyGCMS analysis of the AUR preparation from this experiment clearly showed that lignin biomarkers comprised only a small fraction of AUR, with fatty acids, cutin and other materials contributing. We assume that unknown changes in the chemistry of the AUR fraction, most presumably an accumulation of fatty acids, cutin and microbial products, have led to the above-mentioned large increase in this fraction with progressing litter decomposition. Correlations presented in the paper were nonetheless calculated for solvent/acid extraction data of cellulose and AUR.

Elemental stoichiometry of the initial litter used in this experiment has already been described elsewhere (Wanek et al., 2010). Litter chemistry including elemental contents and ratios, pH and concentrations of NH_4^+ , NO_3^- , PO_4^{3-} and macro- and micronutrients of the initial litter are given in Table 2. Differences in C:N_{lit} , C:P_{lit} and N:P_{lit} were significant for the four litter types (Table 1) and these differences persisted across all three harvests.

Table 2
Litter element contents, elemental stoichiometry, pH and concentrations of ammonium, nitrate and phosphate and macro- and micronutrients in four beech litter types (A, Achenkirch, K, Klausenleopoldsdorf, O, Ossiach, S, Schottenwald) measured after 2 weeks of decomposition. Given are means (\pm SE) ($n = 5$).

	A	K	O	S
pH	6.5 (± 0.0)	6.5 (± 0.0)	6.4 (± 0.1)	6.4 (± 0.1)
C _{lit} (mg g ⁻¹ d.w.)	508 (± 4)	494 (± 5)	481 (± 4)	489 (± 3)
N _{lit} (mg g ⁻¹ d.w.)	8.79 (± 0.12)	9.40 (± 0.13)	8.03 (± 0.13)	11.71 (± 0.16)
P _{lit} (mg g ⁻¹ d.w.)	0.40 (± 0.01)	0.32 (± 0.01)	0.53 (± 0.01)	0.70 (± 0.01)
C:N _{lit} (mass ratio)	57.9 (± 0.6)	52.6 (± 0.5)	60.0 (± 0.7)	41.8 (± 0.8)
C:P _{lit} (mass ratio)	1282 (± 21)	1548 (± 25)	905 (± 15)	699 (± 9)
N:P _{lit} (mass ratio)	22.2 (± 0.5)	29.5 (± 0.6)	15.1 (± 0.3)	16.8 (± 0.4)
NH ₄ ⁺ (μ g NH ₄ ⁺ -N g ⁻¹ d.w.)	2.54 (± 0.20)	10.39 (± 0.68)	6.70 (± 0.51)	13.18 (± 1.21)
NO ₃ ⁻ (μ g NO ₃ ⁻ -N g ⁻¹ d.w.)	2.24 (± 0.07)	2.33 (± 0.07)	2.09 (± 0.09)	3.12 (± 0.16)
PO ₄ ³⁻ (μ g PO ₄ ³⁻ -P g ⁻¹ d.w.)	16.9 (± 2.3)	17.1 (± 3.8)	37.5 (± 4.6)	77.9 (± 26.3)
K ⁺ (mg g ⁻¹ d.w.)	2.58 (± 0.02)	5.36 (± 0.04)	2.08 (± 0.01)	5.48 (± 0.04)
Ca ²⁺ (mg g ⁻¹ d.w.)	13.3 (± 0.1)	12.6 (± 0.2)	16.3 (± 0.1)	12.3 (± 0.2)
Mg ²⁺ (mg g ⁻¹ d.w.)	2.72 (± 0.02)	1.40 (± 0.01)	1.96 (± 0.01)	1.50 (± 0.01)
Fe (μ g g ⁻¹ d.w.)	209 (± 2)	208 (± 4)	453 (± 12)	192 (± 4)
Mn (μ g g ⁻¹ d.w.)	172 (± 3)	1429 (± 10)	776 (± 9)	2137 (± 51)
Zn ²⁺ (μ g g ⁻¹ d.w.)	30.6 (± 0.4)	33.0 (± 0.3)	35.8 (± 1.0)	42.2 (± 0.6)

3.2. Gross rates of glucan depolymerization and glucose consumption

Gross rates of glucan depolymerization and glucose consumption (Fig. 2) varied significantly between harvests and litter types (Table 1). Glucan depolymerization showed a different time course for each of the four litter types. After two weeks, litter from Ossiach had the highest rates of all four litter types (1390 μ g C g⁻¹ d.w. d⁻¹), but rates decreased to intermediate levels after three months (507 μ g C g⁻¹ d.w. d⁻¹). The Achenkirch litter, which had the lowest rates of all four litter types in the beginning (153 μ g C g⁻¹ d.w. d⁻¹), showed a slight increase in glucan depolymerization until reaching levels similar to those of the other litter types six months after inoculation (291 μ g C g⁻¹ d.w. d⁻¹). Glucan depolymerization in Schottenwald litter was relatively low in the beginning (433 μ g C g⁻¹ d.w. d⁻¹), then peaked after three months, where Schottenwald litter had the highest rates of all four litter types (963 μ g C g⁻¹ d.w. d⁻¹), and finally decreased to levels similar to the beginning (281 μ g C g⁻¹ d.w. d⁻¹). Glucan depolymerization rates of litter from Klausenleopoldsdorf were intermediate and constant throughout the experiment (between 366 and 562 μ g C g⁻¹ d.w. d⁻¹). After six months of incubation, differences in glucan depolymerization rates between litter types were no longer significant.

Gross rates of glucose consumption (Fig. 2) also showed significant differences between harvests and litter types (Table 1). Three of the four litter types (Ossiach, Schottenwald and Klausenleopoldsdorf) showed a strong decrease in glucose consumption rates over time with exception for litter from Achenkirch, which had generally very low rates at all time points that did not change over time. After two weeks of decomposition, glucose consumption rates of litter from Klausenleopoldsdorf, Ossiach and Schottenwald were at their highest levels (between 1690 and 2580 μ g C g⁻¹ d.w. d⁻¹), being approximately 10-fold higher than the rates of Achenkirch litter (237 μ g C g⁻¹ d.w. d⁻¹). After three months glucose consumption rates had declined until reaching very low levels at the end of the incubation after six months (between 331 and 574 μ g C g⁻¹ d.w. d⁻¹), varying only slightly between the four litter types.

3.3. Glucose concentration and mean residence time

Glucose concentrations (Fig. 2) in litter from Klausenleopoldsdorf, Ossiach and Schottenwald were highest after two weeks (between 160 and 354 μ g g⁻¹ d.w.), then decreased by 80% after

three months (ranging from 25.3 to 56.5 μ g g⁻¹ d.w.) and did not change further. Litter from Achenkirch exhibited very low glucose concentrations from the beginning (16.7 μ g g⁻¹ d.w.), which did not change throughout the experiment. Mean residence times (MRT) for glucose (Fig. 2), calculated by dividing the pool size by the mean of influx and efflux rates, were highest at harvest one (0.23–0.82 d) and declined toward harvests two (0.11–0.16 d) and three (0.08–0.16 d), demonstrating an accelerating turnover of the glucose pool with declining glucose concentration.

3.4. Correlations between glucan depolymerization, glucose consumption, litter stoichiometry and litter chemistry

Linear regressions were performed to explore the influence of litter chemistry and elemental stoichiometry on glucan depolymerization and glucose consumption rates. Correlation coefficients are shown in Supplementary Table 1 for harvest one and Supplementary Table 2 for the combined harvests two and three. We decided to split the dataset, examining harvest one ($n = 20$) separately from the combined data of harvests two and three ($n = 40$), because our data suggested that initially starch was the main glucan being degraded, while at later stages of the experiment cellulose was the primary substrate for glucan depolymerization. This was indicated by a correlation between litter starch content and glucan depolymerization at harvest one ($r = 0.63$, $p < 0.05$) and a rapid decrease of starch content in the first three months of our experiment. In contrast, at harvests two and three glucan depolymerization was no longer related to starch content, but instead showed a very robust relationship (Fig. 3, $r = 0.80$, $p < 0.001$) to cellulase activity. Additionally, cellulases were almost not present at harvest one (Fig. 4), further corroborating starch as the main substrate for glucan depolymerization at early stages of decomposition.

After two weeks of decomposition litter stoichiometry was not correlated with glucan depolymerization and glucose consumption rates. For the combined data of harvests two and three, however, we found significant relationships of C:N_{lit} (Fig. 5) with glucan depolymerization ($r = -0.44$, $p < 0.01$) and glucose consumption ($r = -0.47$, $p < 0.01$). The relationship was also significant for harvest two when investigated independently ($p < 0.001$, $r < -0.72$), but not for harvest three ($p < 0.25$). C:P_{lit} and N:P_{lit} were not correlated with glucan depolymerization and glucose consumption rates. At harvest one, C:N_{lit} was negatively correlated with glucose mean residence time ($r = -0.86$, $p < 0.001$). At harvests two and three we found significant positive relationships

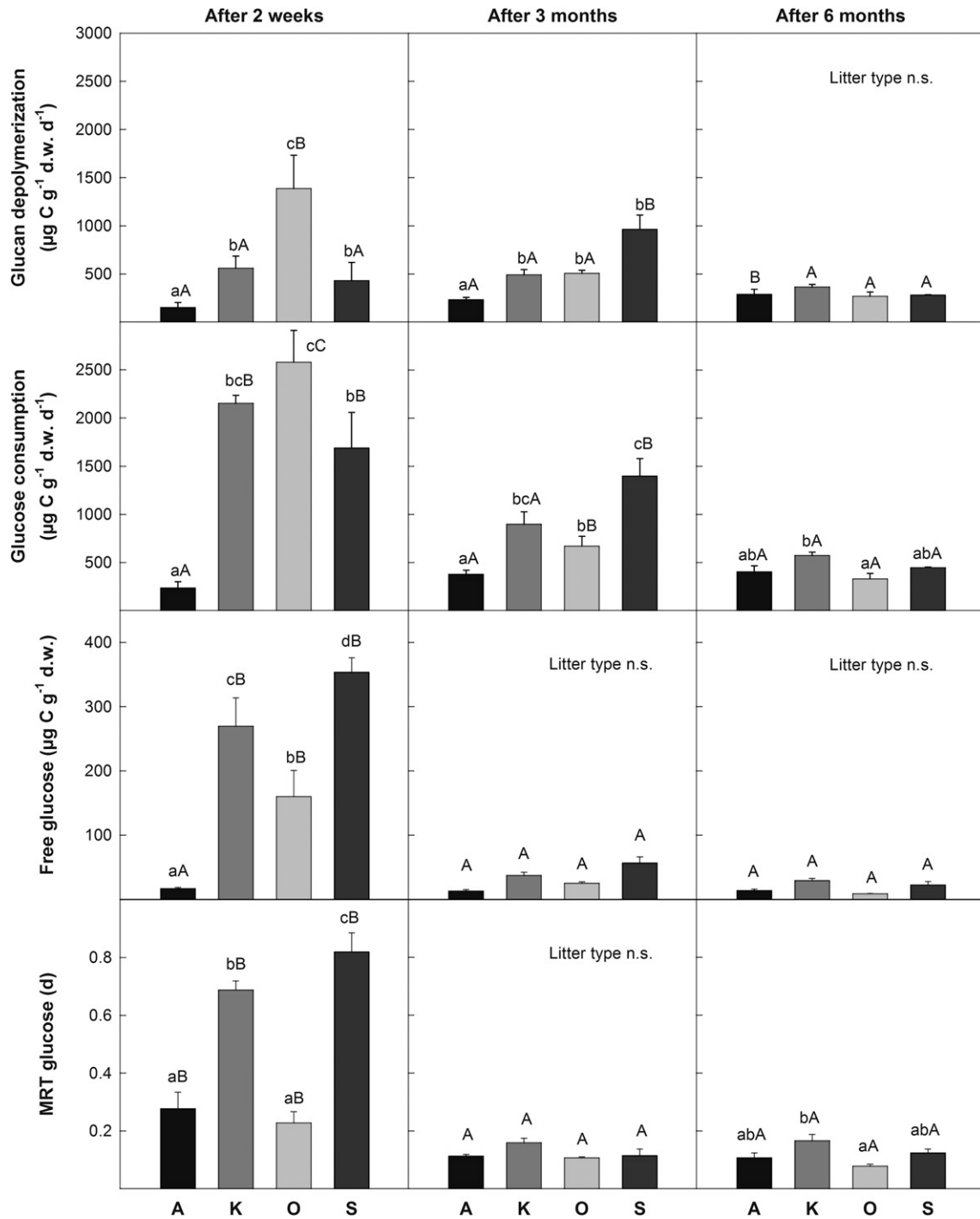


Fig. 2. Gross rates of glukan depolymerization and glucose immobilization and concentrations and mean residence times (MRT) of free glucose at the three sampling time points (after 2 weeks and 3 and 6 months of decomposition, respectively) of the litter decomposition experiment (A, Achenkirch; K, Klausenleopoldsdorf; O, Ossiach; S, Schottenwald). Given are means of 5 mesocosms \pm SE. Lower case letters indicate significant differences between litter types, upper case letters indicate significant differences between harvests (two-way ANOVA followed by Fisher's LSD, $p = 0.05$).

of MRT Glc with C:P_{lit} ($r = 0.52$, $p = 0.001$) and N:P_{lit} ($r = 0.62$, $p < 0.001$).

The pattern of correlations between litter chemistry and glukan depolymerization also changed over time: after two weeks of decomposition, glukan depolymerization was positively correlated with starch content ($r = 0.63$, $p < 0.05$), and negatively with cellulose ($r = -0.65$, $p = 0.01$) and AUR ($r = -0.55$, $p < 0.05$) content. After three months, however, glukan depolymerization

was not correlated with starch, cellulose or AUR concentration, but we found significant negative relationships of glukan depolymerization with litter cellulose:N ($r = -0.40$, $p < 0.05$) and AUR:N ($r = -0.38$, $p < 0.05$) ratios. Glucose concentration was not correlated with glukan depolymerization and glucose consumption rates at harvest one, but after three and six months we found highly significant positive relationships of glucose concentration with glukan depolymerization ($r = 0.73$, $p < 0.001$) and glucose

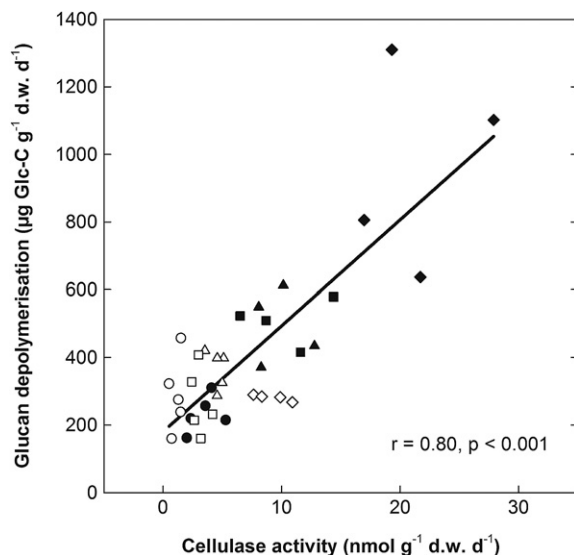


Fig. 3. Linear regression of gross glucan depolymerization and potential cellulase activity. Shown are combined data of four litter types (●, Achenkirch; ▲, Klausenleopoldsdorf; ■, Ossiach; and ◆, Schottenwald) and two time points (black, 3 months and white, 6 months of litter decomposition) ($n = 40$). Letters indicate correlation coefficients (r) and p -values.

consumption ($r = 0.90$, $p < 0.001$) (Fig. 6). Glucose mean residence time was negatively correlated with starch content ($r = -0.75$, $p < 0.01$) and cellulose:N ratios ($r = -0.71$, $p < 0.01$) at harvest one, but we did not find any correlations between litter chemistry and MRT Glc after three and six months.

Cellulases (Fig. 4) were only detectable three and six months after inoculation, where they were negatively correlated with C:N_{lit} ($r = -0.55$, $p < 0.001$) and C:P_{lit} ($r = -0.45$, $p < 0.01$) as well as with cellulose:N ($r = -0.52$, $p = 0.001$) and AUR:N ($r = -0.55$, $p < 0.001$). Furthermore, we found close positive relationships of cellulases with lignolytic peroxidases ($r = 0.58$, $p < 0.001$) and phenolox- idases ($r = 0.63$, $p < 0.001$). Lignin-degrading enzymes (Fig. 4) were present at all three harvests and were generally very closely related ($r = 0.90$, $p < 0.001$).

Glucose consumption was strongly positively related to glucan depolymerization (Fig. 7) at harvest one ($r = 0.76$, $p < 0.001$) and harvests two and three ($r = 0.94$, $p < 0.001$). However, we found no correlation whatsoever between glucose consumption and respiration rate.

4. Discussion

The aim of our study was to develop a new isotope pool dilution method, which enabled us to closely examine the influence of litter quality and elemental stoichiometry on rates of glucan depolymerization and glucose consumption during early stage litter decomposition (Fig. 8).

4.1. Rationale of experimental set-up

To test this we used an experimental set-up that was rather artificial: we sterilized four different beech litter types differing in elemental and biochemical composition, re-inoculated them with a beech litter/soil suspension from one site to obtain a uniform initial microbial metacommunity on all four litter types, and incubated the litter in the laboratory for six months. Elemental variation can be large in litter of one plant species collected from different sites while macromolecular composition (cellulose, lignin content) is expected to vary less (see Results). Although the process

of sterilization and re-inoculation destroyed the native microbial community of the litter, we deliberately chose this approach because it allowed us to observe the development of distinct microbial communities and the related process dynamics based on differences in litter quality only, excluding site-specific differences in microbial inocula, soils, temperature and humidity. Our results may be difficult to extrapolate to natural environments, but our simplified, controlled experimental approach allowed us to investigate basic relationships between resource quality (elemental stoichiometry, C chemistry), process dynamics and the related enzymatic activities. This simplified controlled experimental set-up together with a newly developed isotope pool dilution assay allowed us for the first time to dissect the interactions between resource quality, enzyme activity and gross rates of glucan depolymerization and glucose consumption.

4.2. Temporal dynamics of glucan depolymerization

We demonstrate that litter composition, i.e. the availability of starch and cellulose, plays an important role in the temporal regulation of glucan depolymerization. Initially, glucan depolymerization rates were very high and positively correlated with starch content, suggesting that starch was the primary substrate for glucan depolymerization at this early stage of decomposition. This finding was supported by the lack of cellulolytic enzyme activity at this time point and the decrease of starch concentration in the litter in the first three months of decomposition. Furthermore, glucan depolymerization was correlated with mass loss at the first harvest, suggesting that glucan decomposition contributed substantially to litter mass loss at this stage. After three months, the correlation between glucan depolymerization and starch content diminished, in line with the often reported rapid degradation of starch in the first weeks of litter decomposition (e.g., Berg and McClaugherty, 2008). Amylase activity was not measured in our experiment, so we cannot directly link glucan depolymerization to amylase activity. However, amylase activity in decaying litter has previously been observed to decrease after the first weeks of decomposition (Fioretto et al., 2005; Papa et al., 2008). In addition, during the rapid depletion of the starch pool, glucan depolymerization rates declined and thereafter showed a strong linear correlation with cellulase activity. We therefore assume that glucose production three and six months after inoculation derived mostly from cellulose degradation. Nevertheless, we found no correlation between glucan depolymerization and cellulose content at harvests two and three; we therefore assume that cellulose depolymerization was enzyme-limited but not substrate-limited at this stage. These findings are also supported by the observation that cellulose content hardly changed over time. As cellulose is present in high concentrations in plant litter (Osono and Takeda, 2005; Papa et al., 2008; Pauly and Keegstra, 2008; Perez et al., 2002; Preston et al., 2009), a cellulose-limited situation would only be encountered at more advanced stages of degradation (Herman et al., 2008). Furthermore, we found a negative relationship between glucan depolymerization and cellulose:N ratio, pointing toward a N-limitation of the glucan depolymerization process (see below).

4.3. Stoichiometric controls of glucan depolymerization

The way how litter stoichiometry influenced glucan depolymerization rates changed over the course of our experiment. Two weeks after inoculation of the litter, when starch was the main substrate for glucan depolymerization, no relationship of glucan depolymerization with either C:N_{lit}, C:P_{lit}, N:P_{lit}, or N and P content of the litter was found. We therefore assume that litter N or P content did not notably influence glucan degradation at this early

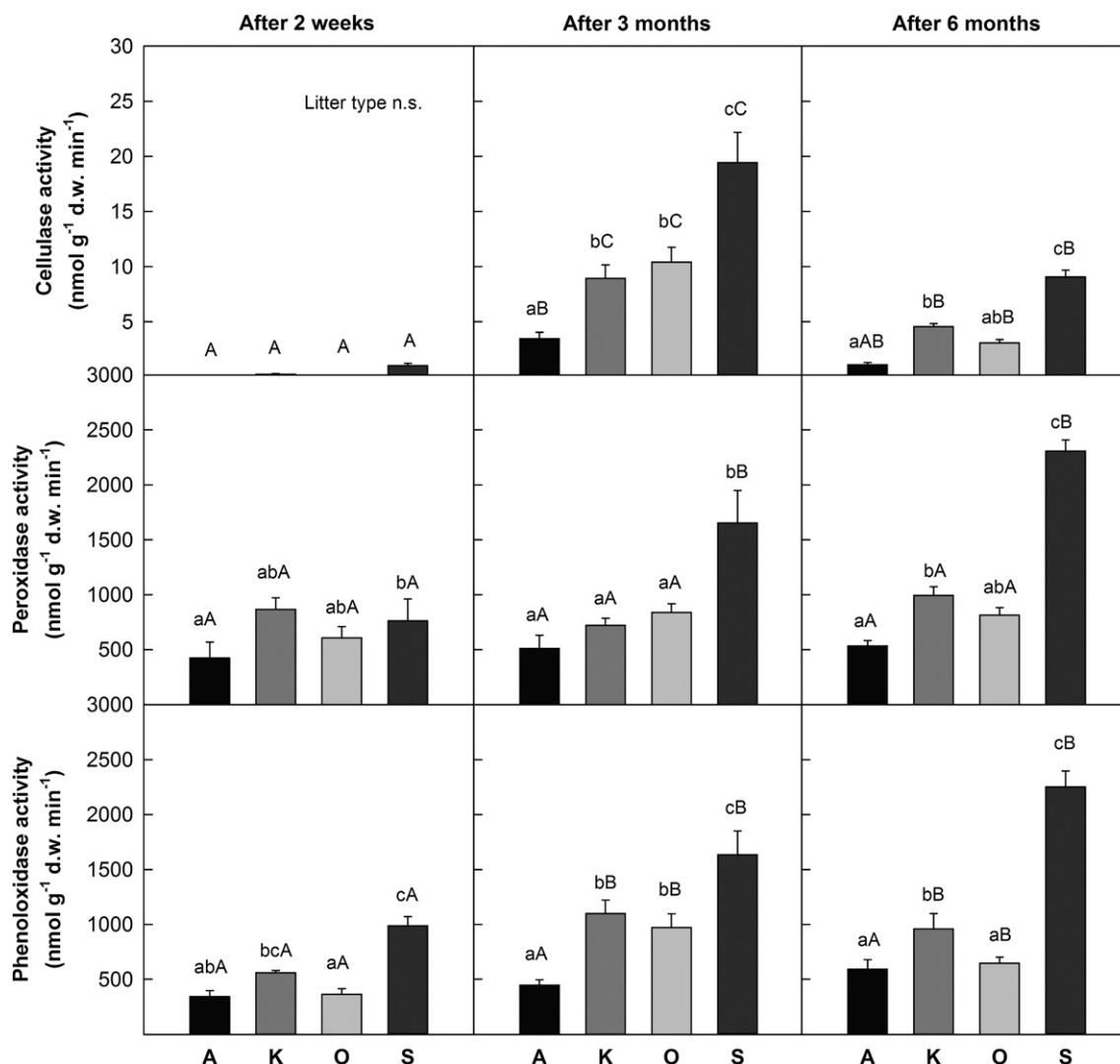


Fig. 4. Potential activities of cellulases, peroxidases and phenoloxidases after 2 weeks and 3 and 6 months of litter decomposition (A, Achenkirch; K, Klausenleopoldsdorf; O, Ossiach; S, Schottenwald). Activities are given in nmol converted substrate (MUF- β -D-cellobioside for cellulase and L-3,4-dihydroxyphenylalanine 'DOPA' for peroxidase and phenoloxidase determination) per gram leaf dry weight per minute. Given are means of 5 mesocosms \pm SE. Lower case letters indicate significant differences between litter types, upper case letters indicate significant differences between harvests (two-way ANOVA followed by Fisher's LSD, $p = 0.05$).

stage of decomposition, probably because labile N or P containing substances (e.g., amino acids, RNA or inorganic N and P) were still available. At later stages of our experiment, however, when cellulose became the primary substrate, glucan depolymerization was correlated with $C:N_{lit}$ and N_{lit} , indicating that glucans were preferably decomposed in litter with a high N content. Furthermore, we found negative relationships between cellulase activity and $C:N_{lit}$ and $C:P_{lit}$, probably indicating that low $C:N_{lit}$ and $C:P_{lit}$ ratios resulted in increased production of cellulolytic enzymes (Fig. 8). These findings are in line with the previously reported accelerated degradation of cellulose in leaf litter (Allison et al., 2010; Berg and Matzner, 1997; Sjöberg, 2004) and increased activities of cellulases in litter (Carreiro et al., 2000; Keeler et al., 2008; Saiya-Cork et al., 2002; Weand et al., 2010) after N fertilization. Sinsabaugh et al. (2009) pointed out that the ratio of activities of C- to nutrient-acquiring enzymes is relatively constant across scales and ecosystems with the mean C:N:P ratio of global extracellular enzyme activities approximating 1:1:1, reflecting the equilibrium between microbial growth efficiency and the elemental compositions of substrate and microbial biomass. Considering this premise and assuming that the differences in $C:N_{lit}$ that we observed in our

experiment were caused by variation in N_{lit} not C_{lit} , we propose that in litter with high N content more resources were allocated to C-acquiring enzymes like cellulases, resulting in higher glucan depolymerization rates in litter with low $C:N_{lit}$ (Fig. 8). This was also supported for harvest 2 and 3 by the negative correlation between glucan depolymerization and $C:N_{imbal}$, i.e. the imbalance between microbial and litter stoichiometry (the ratio of $C:N_{lit}$ to $C:N_{mic}$). A high $C:N_{imbal}$ implies that $C:N_{lit}$ is high compared to $C:N_{mic}$, thus microbes experience N-limitation. Otherwise, if $C:N_{lit}$ is very close to $C:N_{mic}$, $C:N_{imbal}$ is low and microbes might be rather C-limited. The negative relationship between glucan depolymerization and $C:N_{imbal}$ therefore suggests that glucans, which are rich in C and only contain minute amounts of associated N in plant cell walls (Brett and Waldron, 1996) or starch granules, are depolymerized to a greater extent if the microbial community is sufficiently supplied with N.

4.4. Enzymatic relationships

Examining the relationships between enzymatic activities, we found close relationships between cellulases and oxidative

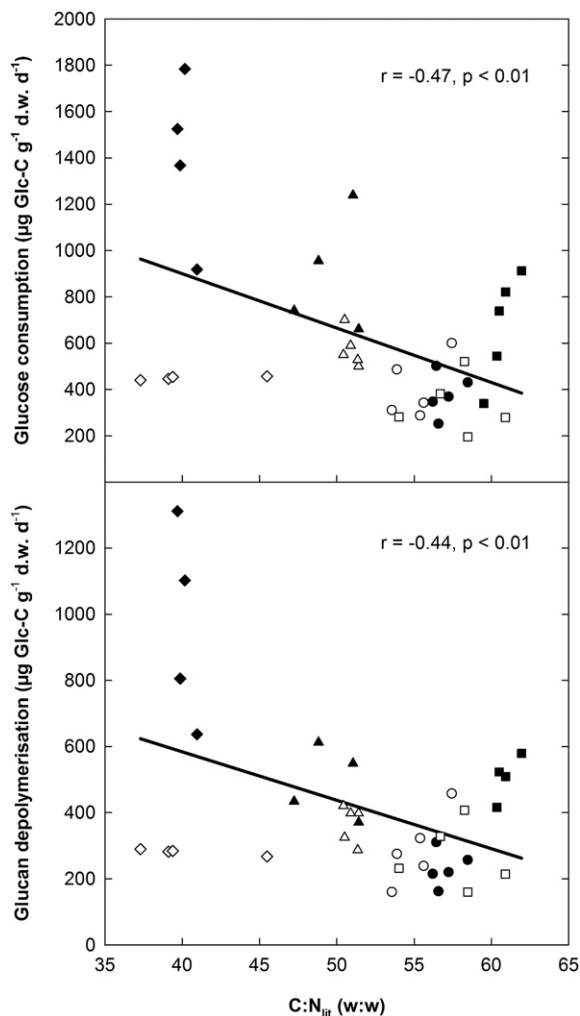


Fig. 5. Influence of litter C:N ratio ($C:N_{lit}$) on rates of glucan depolymerization and glucose consumption. Shown are combined data of four litter types (●, Achenkirch; ▲, Klausenleopoldsdorf; ■, Ossiach; and ◆, Schottenwald) and two time points (black, 3 months and white, 6 months of litter decomposition) ($n = 40$). Letters indicate correlation coefficients (r) and p -values.

enzymes (peroxidases and phenoloxidas) involved in lignin degradation after three and six months of decomposition, probably indicating a synergistic action between cellulose- and lignin-degrading enzymes. This was confirmed by the correlation between glucan depolymerization and the lignin-degrading enzymes peroxidase and phenoloxidase. As cellulose fibers are tightly associated with lignin molecules (Perez et al., 2002), a concerted degradation of the lignocellulose complex seems plausible as previously suggested (Berg and McClaugherty, 2008; Cooke and Whipps, 1993; Herman et al., 2008; Perez et al., 2002; Romani et al., 2006).

4.5. Microbial consumption of glucose

Gross glucose consumption was highly correlated with glucan depolymerization, although both processes are thought to have different controls: glucan depolymerization may be driven by the activities of extracellular enzymes secreted into the soil environment whereas glucose is taken up directly by soil microorganisms. However, both processes are intrinsically linked by the demand of microbes for glucose and the resource allocation to enzymes that

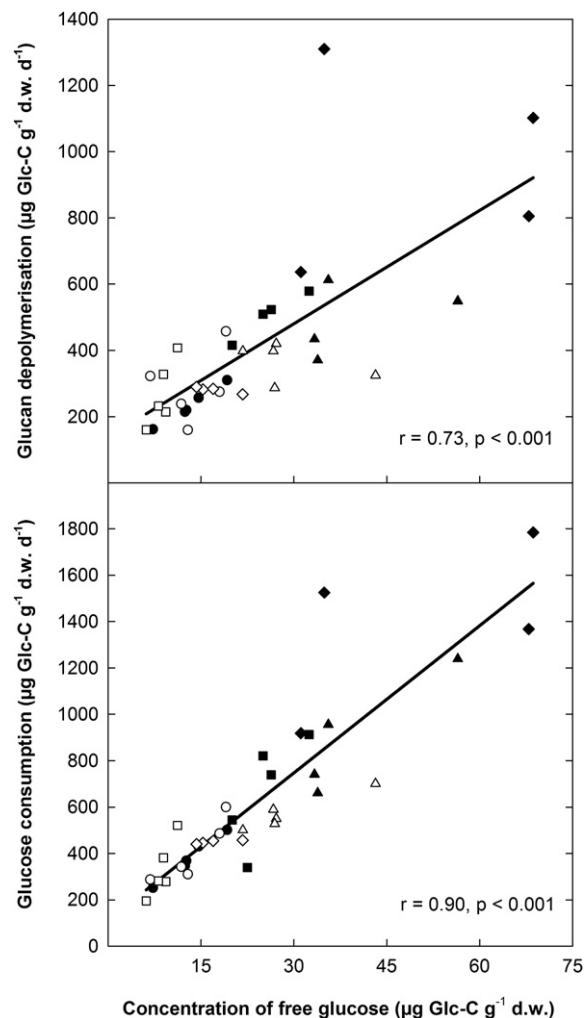


Fig. 6. Linear regression of glucan depolymerization and glucose consumption with free glucose concentration in beech litter. Shown are combined data of four litter types (●, Achenkirch; ▲, Klausenleopoldsdorf; ■, Ossiach; and ◆, Schottenwald) and two time points (black, 3 months and white, 6 months of litter decomposition) ($n = 40$). Letters indicate correlation coefficients (r) and p -values.

degrade glucans and thus liberate glucose (Sinsabaugh et al., 2009). Our results suggest that extracellular glucan depolymerization and microbial glucose consumption are tightly co-regulated and that glucan depolymerization is a 'fine-tuned' process meeting the momentary microbial demand for glucose. As the production of extracellular enzymes is highly nutrient- and energy-demanding and therefore expensive for microorganisms, it is very likely that cellulolytic enzymes are only produced to an extent necessary to satisfy the microbial demand for C (Schimel and Weintraub, 2003; Sinsabaugh et al., 2008). In support of this hypothesis we found significant relationships of glucose consumption with $C:N_{lit}$ and with N_{lit} , suggesting that a higher N content of the substrate resulted in an improved N supply of the microbial community and a subsequently higher microbial demand for C. Furthermore, we found significant correlations between glucose consumption and glucose concentration of the litter, indicating that the demand of the microbial community for glucose was not saturated. As glucose is an easily accessible carrier of C and energy, it should be readily taken up by microbes. Nevertheless, we did not find any correlation between glucose consumption and respiration throughout the experiment, suggesting that glucose was not the only substrate used for microbial respiration. A correlation between glucose

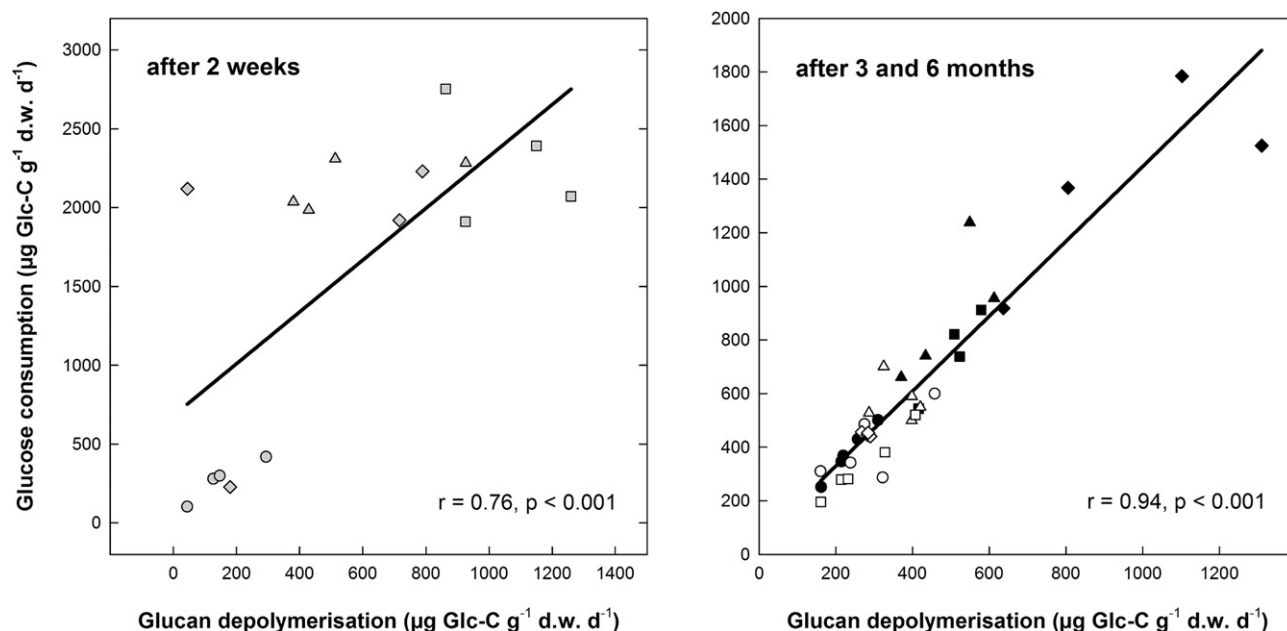


Fig. 7. Linear regressions of glucose consumption and glucan depolymerization after 2 weeks ($n = 20$) and after 3 and 6 months (combined data, $n = 40$). Shown are data of four litter types (●, Achenkirch; ▲, Klausenleopoldsdorf; ■, Ossiach; and ◆, Schottenwald) and two time points (black, 3 months and white, 6 months of litter decomposition). Letters indicate correlation coefficients (r) and p -values.

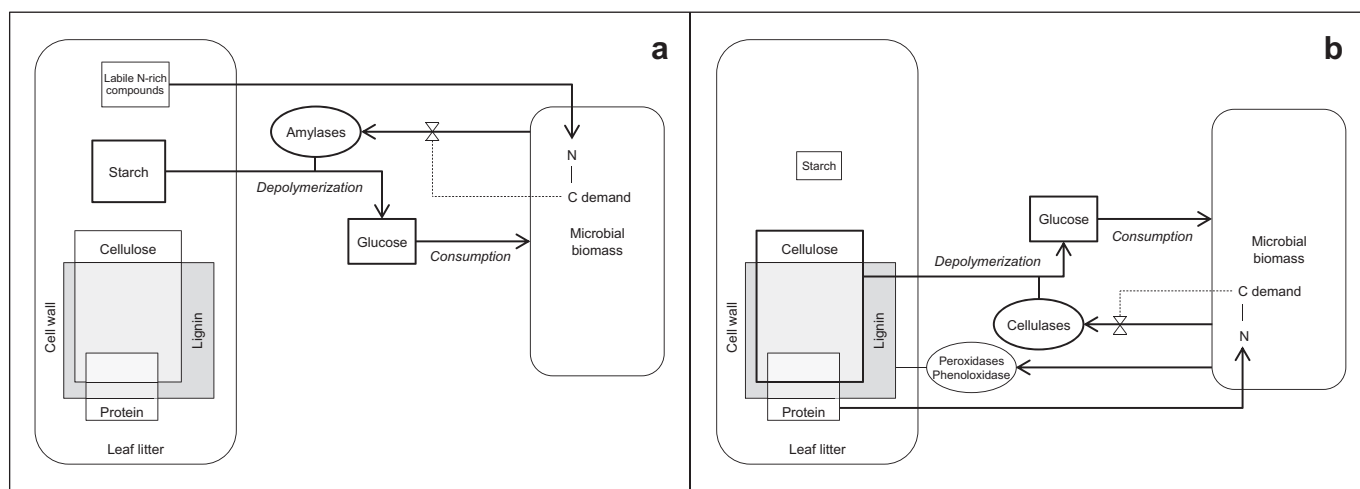


Fig. 8. Conceptual diagram of sources and controls of glucan depolymerization in decomposing beech litter. At the beginning of decomposition (a), starch is the primary source of glucan depolymerization. At this stage, labile N-rich compounds (e.g., amino acids), which derive from the litter material, serve as easily accessible N-source for the microbial community. After depletion of the starch pool after a few weeks of litter decomposition (b), cellulases are produced and the more recalcitrant cellulose pool in the plant cell walls is decomposed. At this stage of decomposition, labile N-sources are mostly depleted and microbial decomposers have to degrade protein to obtain N. The ratio of lignocellulose (contains most of litter C) to protein (contains most of litter N) corresponds roughly to the total C:N of the litter. Cellulose depolymerization is facilitated by the activity of oxidative lignolytic enzymes like peroxidases and phenoloxidases, which attack the lignocellulose complex in plant cell walls and thus provide access to cellulose fibers for cellulases. In general, production of glucan-degrading enzymes is controlled by the microbial C demand. The C demand, in turn, is related to N supply of the microbial community because microorganisms can only use C for growth and enzyme production if they have N. The C and N supply to microbial decomposers ultimately depends on the amount and availability of C and N present in plant litter. Squares represent substrate and product pools, ellipses represent enzyme pools, arrows represent fluxes, valves represent controls of fluxes. Size of pools is not represented in quantitative relationships.

consumption and respiration would only be expected, if all (or a constant proportion) of the consumed carbon source (i.e., glucose) were actually respired. However, it is likely that a variable part of the C acquired by glucose uptake was assimilated and used for buildup of new microbial biomass.

4.6. Conclusions

In summary our results suggest that plant litter chemistry and elemental stoichiometry constitute important controls of glucose

production and glucose consumption by microorganisms during litter decomposition (Fig. 8). Glucan depolymerization was clearly controlled by the availability of suitable substrates, which changed over the course of experiment: at initial stages of decomposition starch was the primary substrate for depolymerization, while at later stages of decomposition cellulose was the main substrate. Interestingly, potential cellulase activities were highly correlated with glucan depolymerization, indicating that at later stages of litter decomposition glucan depolymerization was controlled by size of the enzyme pool, not by the content and accessibility of

cellulose. We were further able to demonstrate that the N content and C:N ratio of the litter strongly affected glucan depolymerization at later stages of decomposition, suggesting an increased C demand of the microbial community and subsequently an increased resource allocation to C-acquiring enzymes with increasing N availability. These are the first data published to date on gross rates of glucose dynamics in decomposing organic matter, which allowed to discern the controls of gross glucan depolymerization and gross rates of microbial glucose use. The work therefore stands in contrast to previously published studies that either measured net changes in cellulose or other carbohydrate pools during litter decomposition, or focused on potential enzyme activities involved in carbohydrate breakdown. The wider application of isotope pool dilution assays in litter and soil organic carbon decomposition studies will represent a leap forward in our understanding of microbial carbon use, of *in situ* activities of extracellular enzymes and the regulation of microbial partitioning of energy (carbon) into extracellular enzymes, biomass production and respiration.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.soilbio.2012.03.012.

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